and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Biochemical desaturation/elongation of essential fatty acids to polyunsaturated fatty acids.

FIG. 2. Dose-response of GLA supplementation on serum fatty acid levels.

FIG. 3. Effect of GLA supplementation (up to 12 weeks) on fatty acid levels in human serum.

FIG. 4. Dose-response of GLA and metabolites supplementation into neutrophil lipids.

FIG. 5. Effect of GLA supplementation (up to 12 weeks) on fatty acid levels in human neutrophils.

FIG. 6A and FIG. 6B. Incorporation of AA (FIG. 6A) and DGLA (FIG. 6B) into glycerolipid classes of neutrophils.

FIG. 7. Fatty acid release from stimulated neutrophils before and after supplementation.

FIG. 8. Influence of GLA supplementation on leukotriene generation.

FIG. 9. Influence of GLA supplementation on 5-lipoxygenase activity.

FIG. 10. In vitro metabolism of GLA in human neutrophils.

FIG. 11. Metabolism of ^{14}C-DGLA to products by stimulated neutrophils.

FIG. 12. Influence of 15-HETrE on leukotriene generation.

FIG. 13. In vitro metabolism of stearidonic acid in human neutrophils.

FIG. 14. In vitro metabolism of stearidonic acid in human neutrophils.

FIG. 15. Metabolism of GLA by human eosinophils.

[FIG. 16. Influence of borage oil on early and late asthmatic response.]

FIG. [17]16A and FIG. [17]16B. The two in vivo approaches to be used in order to synthesize close structural analogues of AA without affecting circulating AA levels. FIG. [17]16A. GLA supplementation in combination with EPA. FIG. [17]16B. Stearidonic Acid Supplementation.

FIG. [18]17A. Bar graph indicating inhibition of arachidonic acid synthesis in liver cells by Δ^5 desaturase inhibitor, eicosapentaenoic acid.

FIG. [18]17B. Per cent inhibition of arachidonic acid synthesis in liver cells by Δ^5 desaturase inhibitor, eicosapentaenoic acid.

ABBREVIATIONS

AA, 20:4, arachidonic acid; EPA, 20:5 (n-3), eicosapentaenoic acid; LA, 18:2, linoleic acid; EFA, essential fatty acid; PUFA, polyunsaturated fatty acid; GLA, 18:3 (n-6), gammalinolenic acid; DGLA, 20:3 (n-6), dihomogammalinolenic acid; SDA, 18:4 (n-3), stearidonic acid; ω-3 AA, 20:4 (n-3); PC, phosphatidylcholine; PE phosphatidylethanolamine; PL phosphatidylinositol; GPC, sn-glycero-3-phosphocholine; GCRC, General Clinical Research Center; GC/MS, gas chromatography/mass spectrometry; NICI negative ion chemical ionization; TNF, tumor necrosis factor; FMLP, n-formyl-methionine-leucine-phenylalanine; TLC, thin layer chromatography; HPLC high pressure liquid chromatography; LTB_4, leukotriene B_4; LTB_5, leukotriene B_5; LTC_4 leukotriene C_4; PAF, platelet activating factor; HBSS, Hank's Balanced Salt Solution; BALF, bronchoalveolar lavage fluid; EAR, early asthmatic response; LAR, late asthmatic response.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present disclosure provides a dietary strategy, including nutritional supplements, designed to improve or at least partially alleviate symptoms of inflammatory disorders by providing a combination of polyunsaturated fatty acids, preferably in a milk or juice based, good tasting drink. The compositions and methods disclosed herein arose in part from the surprising discovery that human neutrophils lack a Δ^5 desaturase activity, and that, while the use of γ-linolenic acid (GLA) in the treatment of arthritis or other inflammatory conditions leads to an increase in arachidonic acid (AA) in serum phospholipids, this increase does not occur in neutrophils. An alternate and synergistic method of inhibiting neutrophil AA metabolism and preventing serum accumulation of AA in response to increased GLA is also available in light of the present discovery. It was contemplated that stearidonic acid (18:4) would also be elongated in neutrophils to form ω-3 arachidonic acid, which would accumulate due to the lack of a Δ^5 desaturase activity (Δ^5 desaturase produces AA from ω-3 arachidonic acid). This excess of ω-3 arachidonic acid is available, then, to

compete with natural AA (n-6) for enzymes (phospholipase A2 isotypes, cyclooxygenase isotypes, and 5-lipoxygenase) that convert AA to oxygenated metabolites. Concomitantly, ω-3 AA formed within the serum may be converted to eicosapentaenoic acid, possibly further inhibiting the hepatic Δ^5 desaturase, and thereby contributing to the inhibition of accumulation of serum AA.

The present disclosure, thus, represents in part, a defined, three pronged mechanism of decreasing symptoms of inflammatory disorders. A precursor of arachidonic acid, such as GLA, may administered to a subject in order to reduce inflammation, as in conventional treatments. GLA administration to humans has been shown to effectively block AA metabolism, block the synthesis of AA products and mitigate the clinical symptoms of inflammatory disorders. As an additional element, the increase in arachidonic acid that is normally seen in serum fatty acids with administration of GLA may be inhibited by administering a Δ^5 desaturase inhibitor, such as eicosapentaenoic acid (EPA), for example. This combination can be utilized in humans to inhibit Δ^5 desaturation of DGLA to arachidonic acid in serum. Also disclosed herein is the synergistic step of providing for the synthesis of close structural analogs (antagonists) of AA by providing stearidonic acid, a competitive substrate of inflammatory cell elongase activity, which in this case, leads to ω-3 arachidonic acid. Thus, the antagonist of AA metabolism in the neutrophils and other inflammatory cells prevents the synthesis of the eicosanoids responsible for an inflammatory response without a concomitant increase in serum AA.

The described strategy is based on the knowledge that when GLA is administered as a dietary supplement, an endogenous elongase activity in inflammatory cells synthesizes a close analogue of AA, DGLA (FIG. [17]16A). A part of the present disclosure is that certain inflammatory cells cannot further desaturate DGLA to AA because they lack a Δ^5 desaturase. However, in human circulation, GLA becomes elongated to DGLA, and then is further desaturated to AA. This leads to a marked increase in AA level in the circulation as a result of GLA administration. The increased AA in the circulation has been shown to cause potentially detrimental effects such as increased platelet reactivity in humans (Seyberth *et al.*, 1975).

The present invention includes a method of providing high concentrations of GLA to humans without causing a concomitant accumulation of serum AA. Thus, high concentrations of GLA can be administered to humans to synthesize DGLA in inflammatory cells, thereby inhibiting AA metabolism, eicosanoid synthesis and attenuating the signs and symptoms of inflammatory disorders without the significant side effect of circulatory AA accumulation. Specifically in the present

Example 6

Effect of GLA Supplementation on Eosinophil Fatty Acid Composition and Airway Functions

An issue with the GLA data obtained with neutrophils is its relationship to atopic asthma and, in particular, whether the neutrophil has a key role in atopic asthma. While there is evidence that the neutrophil has a role in atopic asthma, previous studies, to date, point to the eosinophil as having a central role. Therefore, it was important to determine how GLA was metabolized by human eosinophils. Thus, eosinophils were isolated from atopic subjects and incubated with GLA as described above. Like the human neutrophils, supplementation of human eosinophils resulted in a marked increase in DGLA but no change in the quantity of AA in eosinophil glycerolipids (FIG. 15). These data reveal that eosinophils have the capacity to take up GLA and rapidly elongate it to DGLA. However, eosinophils do not further desaturate DGLA to form AA.

In a second set of studies, two atopic asthmatics were recruited and challenged with antigens as described above. In both subjects, the concentration of antigen necessary to drop FEV_1 by greater than 20% was established. At a subsequent date, they were each challenged with these respective concentrations of antigen and monitored with spirometry to assess the development of an early and late responses. Each of these subjects were then placed on GLA supplementation for four weeks and then challenged again with the same dose of antigen. The subjects were then placed back on their normal diets for two weeks and then challenged again with the same respective dose of antigen. [FIG. 16 shows the average of the responses of the two subjects at the three challenge periods.] The magnitude of the early response was diminished (when compared to pre -and post GLA supplementation) in both subjects four weeks after GLA supplementation. In contrast, GLA supplementation did not influence the late response.

Additionally, the influence of GLA supplementation in a human model of atopic asthma, on eicosanoid production, bronchial reactivity, and airway cellular influx can be measured as detailed herein. A random order, placebo-controlled crossover design preceded by a control diet "run in" phase study is performed. A crossover design is chosen to keep the number of subjects required for statistical validity as small as possible by minimizing the influence of intersubject variability with regards to the severity of asthma, environmental triggers and exposures, and nature and severity of the late asthmatic response (LAR). Subjects are studied after 3 weeks of a controlled "normal" diet with 25% of calories from fat, after 3 weeks of the "experimental" diet consisting of the "normal" diet supplemented with 4.5 grams (15 capsules/day) of GLA as borage oil, and after 3 weeks of a "placebo" diet consisting of the "normal" diet with 4.5 grams (15 capsules/day) of olive oil. Olive oil

is 70% oleic acid, 13% C16, and 15% C18, (<1%, n-3) fatty acids as triglycerides. Neither oil supplement has either an odor or a taste when in capsule form. The experimental and placebo diets are given in random order. Each 3 week period is separated by a 4-6 week usual diet "washout" period when the diet of the study subjects is not controlled. Preliminary data from this group suggests that 4 weeks is a sufficient time period for abolition of an effect of diet during the preceding study period.

Results

It is contemplated that GLA supplementation and not placebo or "normal" diets will mitigate the response to antigen challenge as measured by the decrements in FEV_1 both immediate and the LAP, and reduce the influx of eosinophils into airways during the LAR. GLA supplementation will also likely attenuate antigen-induced urinary LTE_4 exertion and BALF AA increases.

While the antigen challenge model is capable of detecting a therapeutic effect due to prednisone with a small number of subjects, GLA supplementation may be associated with smaller, though significant, effects that are overlooked using relatively small sample sizes. The trial uses 10 subjects per group. Sample sizes are based on variance estimated and differences reported in the preliminary results. The contemplated sample sizes have a 90% power to demonstrate an effect on pulmonary function (FEV_1) that is at least half the magnitude observed with oral prednisone therapy in the pilot study, at an alpha of 0.05. Asthma is a complex disease process and it is possible that significant effects in some components may be missed by using a model that is not sensitive to these effects. For example, an antigen challenge model would not be the appropriate system in which to detect an impact on neurally-mediated immediate processes (e.g., airway cooling). The effect of GLA supplementation, would, however, suggest that this antigen challenge model is appropriate.

Example 7

Dietary Strategies in Humans Utilizing Endogenous Elongase Activity Within Inflammatory Cells to Synthesize Structural Analogs of AA from Dietary Precursors Without Concomitantly Increasing Levels of Circulating AA

The data suggest there may be two strategies that can be utilized in humans to synthesize analogs of AA in inflammatory cells without concomitant increases in serum AA. The first approach (FIG. [17]16A) is to supplement the diets of humans with a combination of gammalinolenic acid (GLA) and a Δ^5 desaturase inhibitor such as eicosapentaenoic acid (EPA), for example. This

strategy is based on in vitro data in hepatocytes and in vivo data in animals which indicate that EPA is a product inhibitor of the enzyme activity that synthesizes it, the Δ^5 desaturase (Gronn *et al.*, 1992; Dang *et al.*, 1989). The inventor has shown in two volunteers that administering of GLA in combination with EPA will induce a marked accumulation of DGLA in circulation and neutrophil lipids without causing a marked accumulation of AA in serum lipids (which is seen with GLA supplementation in the absence of EPA).

If in vivo administration of EPA is an effective means to block the hepatic Δ^5 desaturase, this combination should furnish a means to provide high concentrations of GLA to humans to synthesize the close structural analog of AA, DGLA, in inflammatory cells. This will have the action of inhibiting AA metabolism and eicosanoid biosynthesis, and attenuating signs and symptoms of inflammatory disorders, without the significant side effect of the accumulation of AA in circulation.

The second approach involves administering the n-3 fatty acid, stearidonic acid, to humans (FIG. [17]16B). This fatty acid is converted (by the endogenous elongase in inflammatory cells) to a structural analog of AA, (ω-3 AA and this product will block AA metabolism and thus have anti-inflammatory effects. There have been several studies over the last few years that have examined the effects of in vivo supplementation with alpha linolenic acid (18:3, n-3) in both humans and animals. Generally, these studies have shown that alpha linolenic acid has only modest anti-inflammatory effects (Nordstrom *et al.*, *Rheumatol. Int.*, 14:231-234, 1995; Larsson-Backstrom *et al.*, *Shock*, 4:11-20, 1995; Clark *et al.*, *Kidney Int.*, 48:475-480, 1995; Shoda *et al.*, J. Gastroenterol., 30(suppl 8):98-101, 1995). However, only a very small portion of alpha linolenic acid is converted to stearidonic acid by the Δ^6 desaturase. In fact, this step appears to be the rate-limiting step in n-3 polyunsaturated fatty acid biosynthesis. As described herein, stearidonic acid supplementation is an efficacious means to block AA metabolism because it bypasses the rate-limiting step (Δ^6 desaturase) and is directly utilized by inflammatory cell elongase activity. A major advantage of stearidonic acid verse GLA (alone) as a supplement is that the elongation/ Δ^5 desaturase product from this precursor is EPA and not AA. Consequently even if EPA accumulates in serum components, it will not have the potential detrimental effects of AA.

Example 8

Inhibition of Delta-5 Desaturase by Eicosapentaenoic Acid in Human Liver Cells

The use of Δ^5 desaturase inhibitors in the practice of the present invention rests, in certain aspects, on the ability of those inhibitors to affect Δ^5 desaturase activity in the hepatic cells of a subject who is receiving GLA or DGLA as a dietary supplement, or especially as a treatment for an inflammatory disorder or condition, for example. As is described elsewhere herein, the DGLA, if taken up by liver cells, or GLA that has been elongated to DGLA undergoes Δ^5 desaturation in hepatic cells to produce arachidonic acid. This desaturation does not occur in immune system cells such as neutrophils, which lack the Δ^5 desaturase activity.

In order to demonstrate that an inhibitor such as eicosapentaenoic acid has the capacity to block Δ^5 desaturase, various concentrations of EPA were added exogenously, along with DGLA, to a human liver cell line, HEP-G2, and the conversion of DGLA to AA was monitored. The results are shown in Figs. [18]17A and [18]17B. The data indicate that EPA caused a dose-dependent inhibition of DGLA conversion to AA with a maximum of 50% inhibition at 50μM. This study illustrates the effectiveness of adding a Δ^5 desaturase inhibitor in conjunction with GLA in order to reduce serum arachidonic acid.

Example 9

Determination of the Accumulation of ω-3 AA from Stearidonic Acid Treatment of Neutrophils Affects the Capacity of Cells to Release AA and Synthesize Eicosanoids

In additional work, the inventor has also demonstrated that human neutrophils rapidly take up stearidonic acid (18:4, n-3) and convert it to ω-3 AA. ω-3 AA is a 20 carbon fatty acid that is a close structural analog of AA (n-6). Thus, ω-3 AA may also serve as a competitive antagonist for AA (n-6) during AA metabolism. The following examples provide details of procedures used to investigate this strategy.

It is not known whether ω-3 AA-containing phospholipids will influence the capacity of PLA_2 isotypes to release AA (n-6) in stimulated neutrophils or whether ω-3 AA will effect enzymes distal to phospholipase A_2 such as 5-lipoxygenase or cyclooxygenase I and II. These issues are readily explored by 'loading' human neutrophils in vitro with ω-3 AA; and then activating the cells.